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Electron microscopy demonstrated that desferrioxamine (DFO) appears to act specifically during the late trophzoite and early schizont stages of P. falciparum by preventing nuclear division. This finding is consistent with inhibition of the iron-dependent enzyme ribonucleotide reductase induced by DFO. Inhibition of this enzyme appears to contribute to the antimalarial effect of iron chelate. Immuno-electron microscopy showed liposome-encapsulated malarial antigen that is phagocytosed by macrophages can enter an intracellular compartment in which some of the antigenic epitopes are not degraded by lysosomal enzymes. This indicates the possible existence of a pathwway in which liposomal contents can bypass lysosomal degradation. PRBC sequestration and cytoadherence of knobs on PRBC to endothelial cells in cerebral vessels were shown in rhesus monkeys infected with P. coatneyi. This indicates that rhesus monkeys infected with P. coatneyi can be used as a primate model for human cerebral malaria.

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FOREWORD

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INTRODUCTION:

The main objective of our work is to function as a basic core electron microscopy facility in collaboration with scientists at WRAIR. In particular we proposed to carry out studies on morphological effects of drugs on various microorganisms by electron microscopy and autoradiography in order to understand the mode of actions of these drugs.

In the past, electron microscopy has been used to investigate the mode of action of chemotherapeutic agents on a variety of microorganisms. Studies of chloroquine, primaquine, pyrimethamine, qinghaosu, pentamidine and WR-163577 have indicated that each compound produces specific changes in particular organelles (1-7). The results obtained by electron microscopy have correlated well with biochemical observations on the action of drugs. Therefore, studies of the morphological effects of drugs on various microorganisms can contribute greatly to our understanding of their mode of action.

Secondly, immuno-electron microscopy has been used to characterize protective antigens of malarial parasites. Techniques of immunology and molecular biology have been used to identify and isolate specific proteins that may be capable of producing protective immunity against malarial parasites. Immuno-electron microscopy has played an important role in identifying specific immune targets and characterizing the precise location and expression of these targets (8,9). Such work will help to develop successful vaccines against microorganisms.

BODY:

During this period, we have performed electron microscopy (standard and immuno-electron microscopy) to investigate the mode of action of antimalarials and localization and characterization of protective malarial antigens in host and parasites. Important findings are described as follows:

a) Stage-specific ultrastructural effects of desferrioxmine on Plasmodium falciparum in vitro.

Desferrioxamine B (DFO), the only chelating agent now available for clinical use, is a naturally occurring trihydroxaminic acid isolated from cultures of Streptomyces pilosus (10). Each molecule of DFO binds a single atom of ferric iron with very high affinity. At present, DFO is used for the treatment of acute iron poisoning and for the therapy of iron overload in patients with refractory anemia who require chronic transfusion therapy (11,12).

The antimalarial activity of DFO has been clearly demonstrated in a series of studies both in vitro and in vivo using the erythrocytic and exoerythrocytic stages of rodent and primate plasmodia (13,14). Antimalarial activity in human infection has also been established in a recent clinical trial in adults with asymptomatic P. falciparum parasitemia. However, neither the mechanism nor the specific site of action of DFO have been determined. Therefore, we examined the

ultrastructural effects of DFO on synchronized cultures of P. falciparum to identify the specific site of action of this drug.

Synchronized cultures of early rings or schizonts were exposed to 100 μM DFO for up to 48 hours and fixed and processed at regular intervals for electron microscopy. Untreated cultures and cultures exposed to DFO saturated with $Fe^{3+}$ were processed at the same time. When DFO was added to synchronized cultures containing early rings, parasites developed normally until the late trophozoite stage, when all growth ceased (Fig. 1). Ultrastructural lesions included the breakdown of the unclear envelope into small membranous fragments and progressive vacuolization of the nucleoplasm (Fig. 2). Other organelles, including food vacuoles and mitochondria, were not affected. Addition of DFO to synchronized cultures of schizonts had similar effects on nuclei of early schizonts, but little or no effect on mature schizonts and segmenters. Erythrocyte invasion by merozoites was able to proceed in the presence of the chelator. These findings support the hypothesis that DFO acts specifically during the late trophozoite/early schizont stages of parasite maturation by preventing nuclear division, and effect consistent with inhibition of the iron-dependent enzyme ribonucleotide reductase. Thus, the morphologic evidence suggests that inhibitions of this iron-dependent enzyme contributed to the antimalarial effect of iron chelation.

b) Phagocytosis of liposomes by macrophages: intracellular fate of liposomal malaria antigen.

Liposomes continue to be useful as models for understanding the disposition patterns of materials ingested by macrophages. The fate of liposome-encapsulated protein in macrophages is of particular interest in immunology. Liposomes are highly effective in the stimulation of immune responses to encapsulated protein antigens (15). It is widely believed that the process of immunological presentation of protein antigen to cells in the immune system involves initial processing of the antigen by specialized antigen presenting cells such as macrophages and B lymphocytes. It is presumed that the ability of liposomes to enhance immune responses is due to natural targeting of liposomes to macrophages. In collaboration with Col. Carl R. Alving and his associates of the Department of Membrane biochemistry, WRAIR, we studied intracellular localization and fate of liposomes and liposome-encapsulated malarial antigen after phagocytosis by macrophages.

In the present study, we performed immunoelectron microscopy to detect the presence of all surface-associated and intracellular liposome-associated malarial antigens. After incubation of macrophages with liposomes containing malarial antigen, vacuoles appeared in the cytoplasm and the

vacuoles contained numerous liposomes that were densely labelled by antibody to malarial antigen. Many of the protein-containing liposomes apparently remained intact with a considerable amount of liposome-associated antigen for at least as long as 6 hours after incubation. Liposomes were mainly located at the peripheries of the vacuoles and often seemed to adhere to old vacuolar membranes. Antigenic epitopes detected by the antibody were often observed in the cytoplasm next to the vacuoles. The original antigen consisted largely of repeats of ASN-ALA-ASN-PRO, and it is possible that fragments containing ASN-ALA-ASN-PRO were recognized by the antibody. However, liposomes were invariably present only within the vacuoles and liposome-like structures were rarely, if ever, present in the cytosol of associated with any other subcellular organelles.

We concluded that liposome-encapsulated protein that is phagocytosed by macrophages can enter an intracellular compartment in which at least some of the antigenic epitopes are not degraded by lysosomal enzymes. Furthermore, although liposomes do enter large vacuoles, at least some of the antigenic epitopes, as detected by a monoclonal antibody, can escape into the cytoplasm. These experiments therefore provide evidence to support the possible existence of a pathway in which lipsomal contents can bypass lysosomal degradation. The occurrence of an intracellular pathway for endocytosed or phagocytosed materials that avoids lysosomes has been

previously hypothesized and such a pathway therefore might be used as a basis for delivery of non-degraded liposomal antigenic protein epitopes to the cytosol and to the cell surface.

c) A study on a primate model for human cerebral malaria

Human cerebral malaria is a pernicious manifestation of infection with P. falciparum. Possible factors contributing to the development of cerebral malaria include the blockage of cerebral microvessels by parasitized erythrocytes (PRBC), deposition of immune complexes in cerebral microvessels, reduced humoral or cell-mediated immune responses and action of tumor necrosis factor (TNF) (16).

Electron microscopy revealed multiple electron dense knobs protruding from the membrane of PRBC seen in the cerebral microvessels of cerebral malaria patients (16-18). These knobs attach via a parasite ligand to receptors on endothelial cells, resulting in the blockage of cerebral microvessels. Recently several investigators reported that host cell molecules such as CD36, thrombospondin (TSP) and intercellular adhesion molecule-1 (ICAM-1) may function as the endothelial cell surface receptors for PRBC. However, there is little information as to whether these molecules actually play a role in cytoadherence of PRBC in vivo. We have demonstrated by immunohistochemistry the presence of CD36, TSP and ICAM-1 on the endothelial cells of cerebral microvessels

of fresh-frozen, non-malarious human brain tissues (17). However, formalin-fixed and paraffin-embedded brain tissues do not stain well with antibodies against these molecules by immunohistochemical methods. Thus, the use of fresh brain tissue from cerebral malarial patients appears to be essential for studies of the potential role of these molecules in the development of cerebral malaria. However, it is difficult to obtain fresh brain tissue from cerebral malaria patients, since they often die in malaria endemic areas where no freezing facilities are available.

P. coatneyi produces knobs on the membrane of PRBC and these PRBC appear to sequester in the vasculature of infected rhesus monkeys (19). There have been no reports, however, indicating whether cytoadherence of PRBC to endothelial cells of cerebral microvessels occurs in vivo. Therefore, in collaboration with Col. Webster, Maj. Brown and Maj. Smith of AFRIMS, Bangkok, Thailand, we studied the pathology of the central nervous system (CNS) of rhesus monkeys infected with P. coatneyi in order to determine whether cytoadherence of PRBC to endothelial cells is a consistent feature of infections with this primate parasite and, if so, whether putative receptors for cytoadherence in human cerebral microvessels such as CD36, TSP and ICAM-1 are also present.

Our study demonstrated PRBC sequestration (Fig. 4) and cytoadherence of knobs on PRBC to endothelial cells in cerebral microvessels of these monkeys (Fig. 5) Cerebral

microvessels with sequestered PRBC were shown by immunohistochemistry to possess CD36, TSP and ICAM-1. These proteins were not evident in cerebral microvessels of uninfected control monkeys. Therefore, our study indicates, for the first time, that rhesus monkeys infected with P. coatneyi can be used as a primate model to study human cerebral malaria. By using this animal model, we may be able to make strategies to develop vaccines to prevent human cerebral malaria.

In addition, we are planning to study morphological effects of antimalarial drugs on sequestered parasitized erythrocytes in order to understand how sequestered PPBC become detached from endothelial cells of cerebral microvessels by various antimalarials. Since this aspect of human cerebral malaria has never been studied in the past, our study will help to understand the mode of action of various antimalarial drugs on human cerebral malaria.

d) The localization of EBA in the erythrocytic stages of P. falciparum

In collaboration with Dr. Kim Lee Sim and Col. David Haynes, Department of Immunology, WRAIR, we have been localizing P. falciparum antigen having molecular weight 175 kDa (EBA) within the parasite by immunoelectron microscopy. In particular, SABRE which

stands for the putative "Sialic Acid Binding Region" on the EBA-175 molecule, has 88 amino acid residues and includes EBA-peptide 4. Dr. Kim Lee Sim and Col. Haynes expressed it as an E. coli product fused to the NS 1 protein of influenza virus. This NS 1 protein acts as a great carrier molecule. Our immunoelectron microscopic data by using antibody against SABRE indicated that this antigen distributes diffusely over the cytoplasm of trophozoites and schizonts of P. falciparum grown in vitro. Since immunofluorescent microscopy demonstrated intense staining at the apical end of segmenters and free merozoites, we are trying now to see if we could localize EBA at some apical end organelles of merozoites.

CONCLUSIONS:

As indicated in the section "Introduction" we have collaborated with scientists from WRAIR and AFRIMS in order to investigate morphological effects of drugs, particularly antimalarials on malarial parasites and hosts. Desferrioxamine (DFO) appears to act specifically during the late trophozcite/early schizont stages of parasite maturation by preventing nuclear division. This finding is consistent with inhibition of the iron-dependent enzyme ribonucleotide reductase induced by DFO. Therefore, inhibitions of this iron-dependent enzyme appear to contribute to the antimalarial effect of iron chelation.

Secondly, we performed immuno-electron microscopy to study intracellular localization and fate of liposomes and liposome-encapsulated malarial antigen after phagocytosis by macrophages, since the ability of liposomes to enhance immune responses is due to natural targeting of liposomes to macrophages. Our study indicated that liposome-encapsulated protein that is phagocytosed by macrophages can enter an intracellular compartment in which at least some of the antigenic epitopes are not degraded by lysosomal enzymes.

Our attempt to make a primate model for human cerebral malaria has been successful. PRBC sequestration and cytoadherence of knobs on PRBC to endothelial cells in cerebral microvessels were demonstrated in rhesus monkeys infected with P. coatneyi. These cerebral microvessels with sequestered PRBC were shown to possess CD36, TSP and ICAM-1. By using this animal model, we are planning

to study morphological effects of antimalarial drugs on sequestered PRBC within cerebral microvessels. In addition, by using this model, we may be able to develop vaccine candidates to prevent human cerebral malaria.

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APPENDIX:

Figure Legend

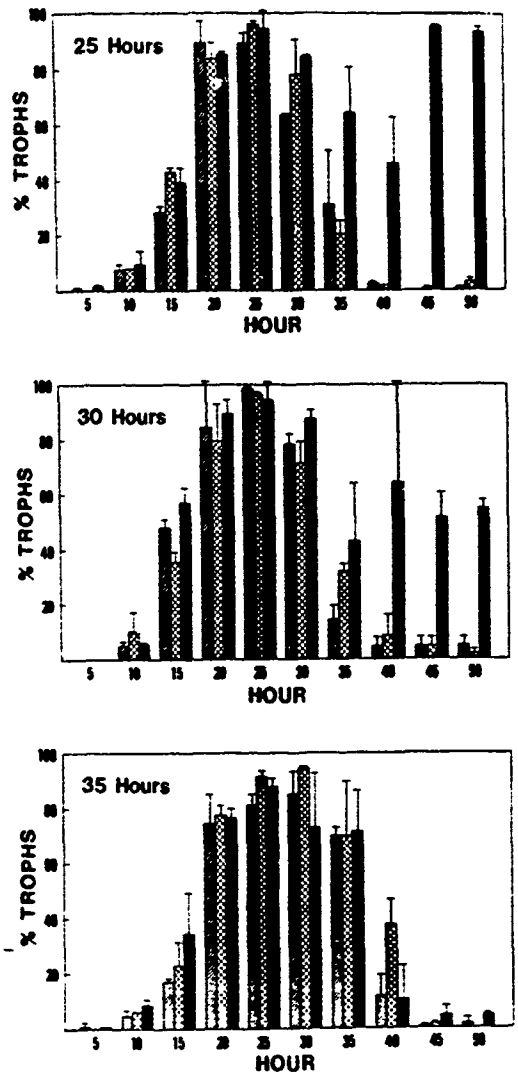
Fig. 1 Composition of synchronized cultures by stage of development during a 48 hour erythrocytic cycle. DFO (■), DFO saturated with iron (▩) or an equal volume of distilled water (▨) was added at 25, 30, and 35 hours after merozoite invasion. Bars represent percentage of parasites in each experimental group that were composed of mature trophozoites. Graph represents the mean of two experiments. Error bars show 1 standard error of the mean. Addition of DFO at 5, 10, 15 and 20 hours after merozoite invasion produced results that were similar to those at 25 hours (top), with almost complete arrest of parasite development at the trophozoite stage. Addition of DFO at 40 and 45 hours after merozoite invasion produced results that were similar to 35 hours (bottom), with little or no effect on parasite development.

Fig. 2 Electron micrographs of young trophozoites of P. falciparum that were exposed to DFO for 14 hours before fixation. Nucleus has a vacuolated area of low electron density that appears to be forming from a large expansion of the nuclear envelope. Note fragments of membrane within the vacuolated area. An

Fig. 3 Immunoelectron micrograph of macrophages. The macrophages were fixed 6 hours after incubation with liposomes containing malaria antigen and the malaria antigen was detected by specific monoclonal antibody (Pf 1B2.2) to the antigen followed by treatment with gold-labeled second antibody. Densely labeled liposomes are present within a membrane bound vacuole. X 50,000.

Fig. 4 Light micrograph showing sequestration of P. coatneyi-infected erythrocytes in a cerebral microvessel of rhesus monkey.

Fig. 5 Electron micrograph showing cytoadherence (arrow) of PRBC knobs to endothelial cells of rhesus cerebral microvessel. X 56,000.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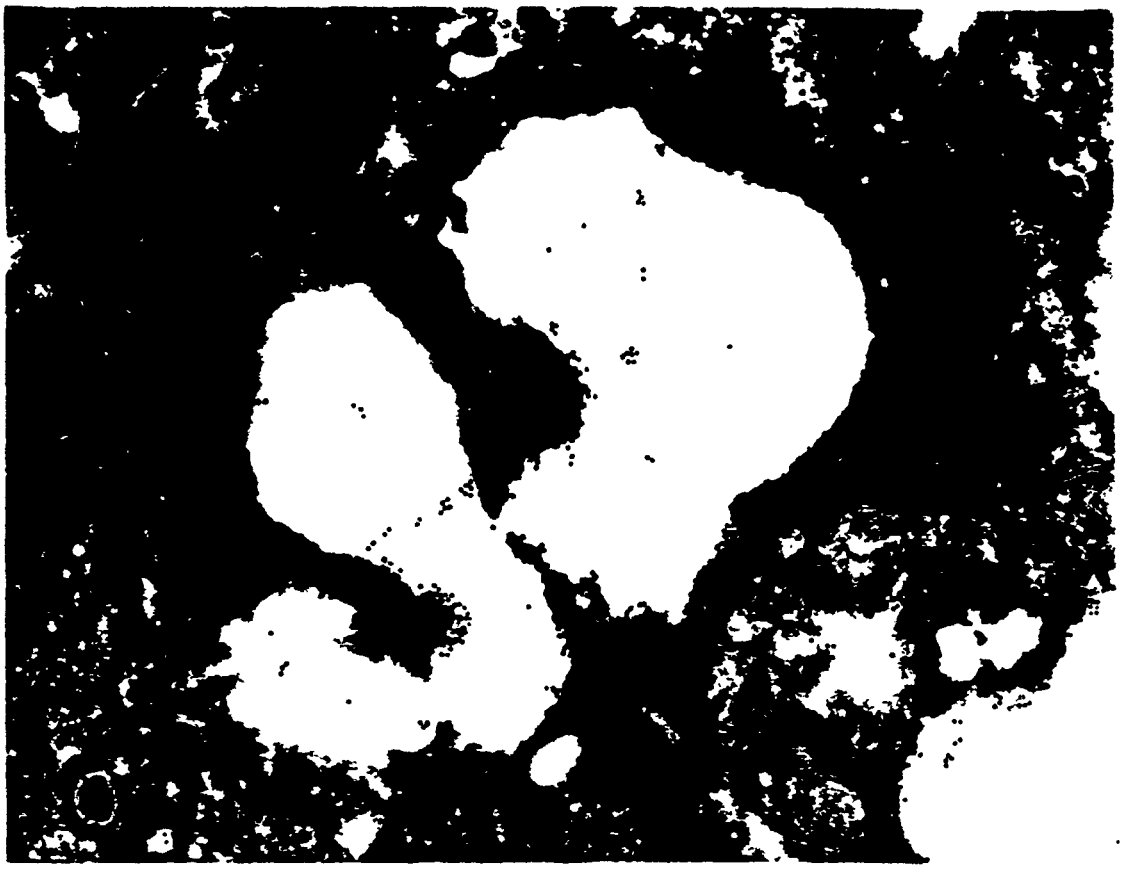

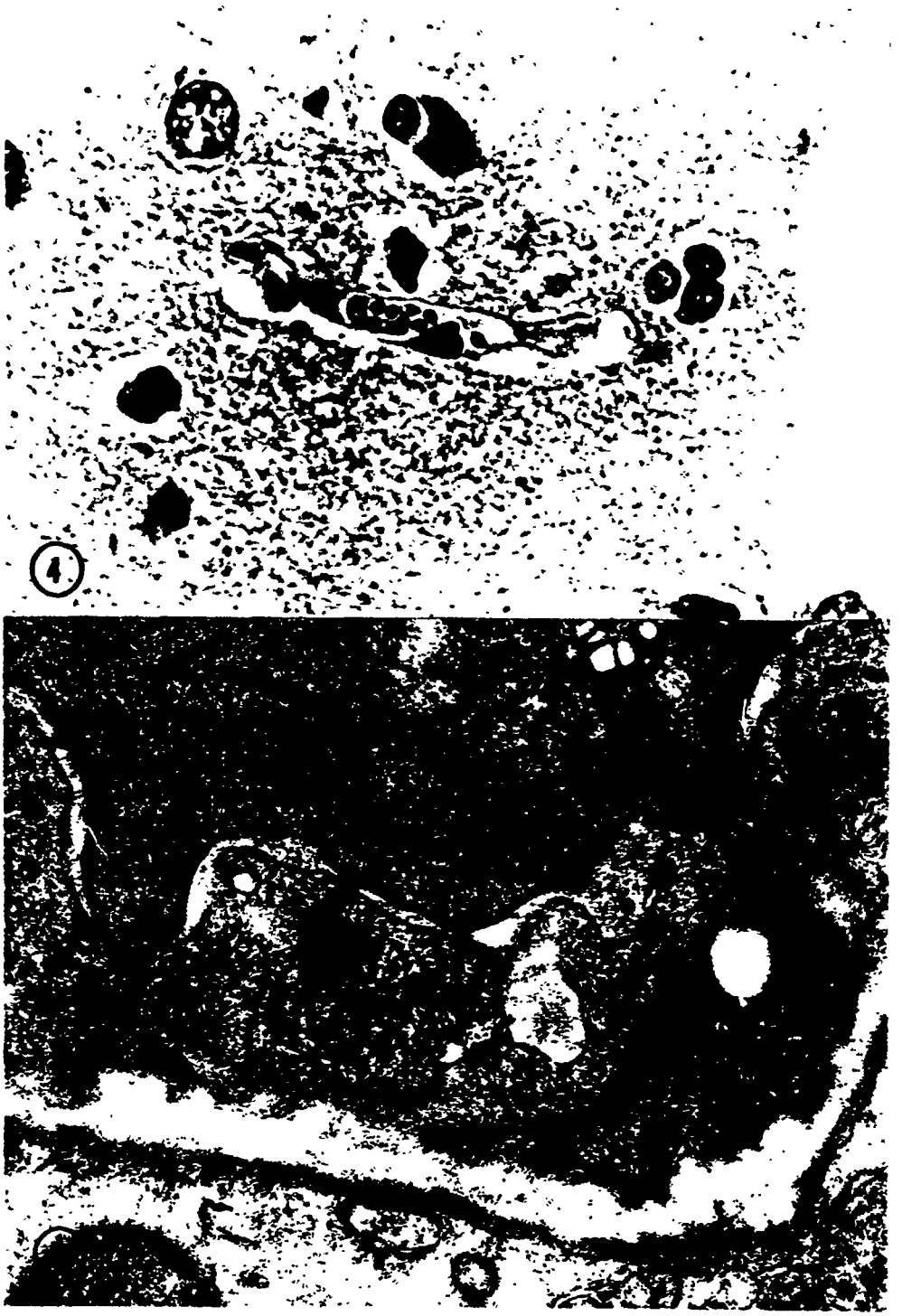
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